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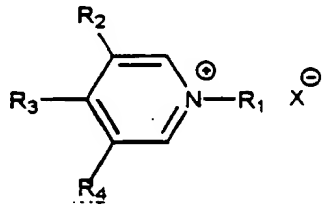
(54) Transportvehicles for macromolecules

(57) The invention relates to new compounds with the general formula I for use as a tool to introduce macromolecules into cells. The invention further relates to compositions for introducing macromolecules into cells, comprising vesicles formed by at least one compound in a solvent.

The macromolecule can be incorporated in the vesicles and/or bound to the vesicles or another aggregate of the new compounds. In a preferred embodiment at least one targeting molecule, for instance a (labelled) antibody, may further be attached to the vesicles.



(I)

EP 0 755 924 A1

Description

The present invention relates to new compounds which are capable of introducing macromolecules into eucaryotic cells.

The introduction of macromolecules, including DNA, proteins and the like, into eucaryotic cells can be carried out in different ways, for instance by means of transport vehicles. Such vehicles introduce a molecule into the cell, for instance by means of endocytosis. The vehicles may bind, but for instance also encapsulate, the molecules to be transported. In the latter case the vehicles are referred to as vesicles. Known vesicles are liposomes which consist of a bilayer of phospholipids.

Liposomes are for instance used to introduce medicines into the cell. It appeared that liposomes are incorporated into the cell both in vivo and in vitro by means of endocytosis (Nandi, P.K. et al. (1986) J. Biol. Chem. 261:16722; Heath, T.D. (1987) Methods Enzymol. 149:111). This means that the largest portion of the material which is incorporated in the cell will ultimately appear in the lyposomal apparatus, where it will be decomposed. Particularly for substances which have their effect in the cytoplasm or the nucleus this is obviously very disadvantageous.

If the substances to be introduced are hydrophilic it will be difficult to introduce them into liposomes. The main portion of the material remains in the aqueous phase. Particularly in case of expensive substances, like probes and many medicines, this is an obvious disadvantage.

To prevent that the substances to be introduced into the cell end up in the cell by means of endocytosis, attempts have been made to use fusogenic phospholipids as transport vehicles. The use of fusogenic phospholipids should result in fusion of the from the fusogenic phospholipids formed vesicles with the cell membrane and thus introduce their contents into the cell. However, such attempts have not proven to be very successful because fusogenic liposomes have a strong tendency to mutually merge instead of fusing with the cell membrane (Fonteijn, T.A.A., Ph.D. Thesis (1992)).

One of the most important applications in which molecules are introduced into a cell is transfection of the (eucaryotic) cell with DNA or RNA. Transfection is being used for studying the function and regulation of genes and proteins, but also for the genetic modification of micro-organisms, plants and animals. There is a large number of artificial techniques which allow DNA to be introduced into a cell, including DNA-micro-injection, DNA-coprecipitation within inorganic salts or with polycations, DNA-encapsulation in liposomes, and making the cell membrane permeable with the aid of chemical or physical means.

A more recent technique involves the use of cationic amphiphilic molecules as transport vehicles. One of the best-known amphiphiles is the quaternairy ammonium amphiphile DOTMA (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride) which in combination with dioleyol phosphatidyl ethanolamine (DOPE), is commercially available with the name Lipofectine™. Both molecules are lipidic (analogues), which form liposomes, which will form complexes with the negatively charged nucleic acids. Supposedly, the liposomes merge with the plasma membrane and introduce in this way nucleic acids into the cell. However, it could also be done by means of endocytosis. The exact mechanism is yet unknown. With the aid of Lipofectine™ the transfection efficiency may be enhanced by a factor of 30 with respect to other known systems, including the classical calcium phosphate precipitation method. However, the disadvantage of Lipofectine™ is its toxicity and therefore it may be difficult or not possible to use it in vivo. Therefore, a demand still remains for other and better transfection methods.

It is the aim of the present invention to provide new cationic amphiphilic compounds, which allow high efficiencies, for the introduction into a cell of nucleic acids and other macromolecules, including for example proteins and medicines.

The aim is achieved by the invention by compounds according to claim 1. Preferred compounds are the subject of the claims 2 to 13. A particularly advantageous compound according to the invention is 1-methyl-4-(19-cis,cis-hepatritiaconta-9,28-dienyl) pyridinium chloride (SAINT-2). The compounds according to the invention are all based on a pyridine ring, which is at one or two positions substituted by a long (ar)alkyl chain. It has been found that with the amphiphiles according to the invention, and particularly with the compound here referred to as "SAINT-2", a transfection efficiency can be obtained which, dependent on the cell type, is at least eight times higher as that of Lipofectine™.

With SAINT-2/DOPE it also proved to be possible to introduce proteins, particularly gelonine (30kD), into the cell. Other cell types, particularly Baby Hamster Kidney (BHK) cells, may be transfected. This is impossible with Lypofectine™ for BHK cells. SAINT-2/DOPE yields even better results with BHK cells than Lipofectine™ with COS-7 cells.

The compounds according to the invention may be synthesized in a well-known fashion. The synthesis will be further illustrated in the examples.

The amphiphiles according to the invention may be used in a large number of applications.

The transport into the cell of nucleic acids and their derivatives is of importance for transfection. The aim of transfection is, for instance, to make proteins or to perform research. Furthermore, transfected nucleic acids, possibly labelled with streptavidine or radioactively labelled, may be used for in situ hybridisation. A more advanced application is to influence gene expression, for instance blocking of genes by antisense strands. Furthermore, gene expression may also be stimulated. Furthermore, the defect genes may be replaced. The latter two applications are of particular importance in gene therapy.

The advantage of compounds according to the invention is that they, as compared to the known transport vehicles, can be used in much lower, non-toxic concentrations. Probably, they also do not cause an immunologic response.

If DNA and/or RNA are to be introduced into a cell the compounds and the nucleic acids have to be mixed in a certain ratio. It has been found that for the known amphiphiles, including DOTMA, there exists an optimum amphiphile concentration (Felgner, P.L. et al. (1987) Proc. Natl. Acad. Sci. USA 84:7413). The transfection efficiency again reduces if a certain amount is exceeded. A comparable situation also holds for the compounds according to the invention.

The cationic amphiphiles according to the invention may also be used to transport negatively charged proteins, including gelonine in particular, into the cell.

The amphiphiles may also be used to transport substances like cytostatics. Lipophilic cytostatics in particular do interact with the compounds according to the invention and may in this way be introduced into the cell very efficiently.

In a preferred embodiment of the invention the transport vehicles may be purposely brought to a specific site by mixing the amphiphiles with a targeting molecule, such as, for instance, an antibody which is directed against an epitope in the neighbourhood of the site where the incorporated substance has to excercise its activity. The antibody is preferably coupled to the amphiphilic compound but it may also be coupled, for instance, through a spacer, to the substance to be transported. In order to facilitate the translocations of DNA or other macromolecules across the cell membrane the compounds according to the invention may also be mixed with a phospholipid or with each other.

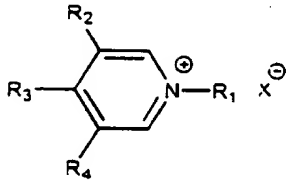
The present invention will be illustrated in further detail with by means of the accompanying examples which are only serve as an illustration and do not limit the scope of the invention.

EXAMPLES

EXAMPLE 1

Synthesis

Compounds with the general structure formula



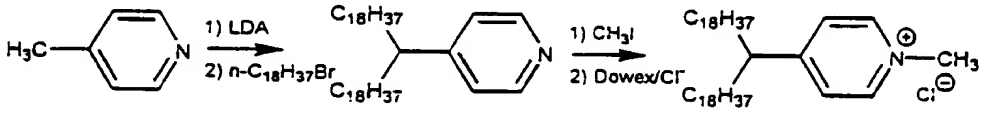
may be divided in a number of groups dependent on their substituents. The synthesis of four of those groups will be given below as an example.

1. 4-Substituted N-alkylpyridinium salts.

1.1. Synthesis of 1-methyl-4-(1-octadecylnonadecyl)pyridinium chloride.

The compound is synthesised according to scheme 1 below as described by E.J.R. Sudhölter in his Ph.D. thesis at the University of Groningen, 1981, page 37.

Scheme 1:

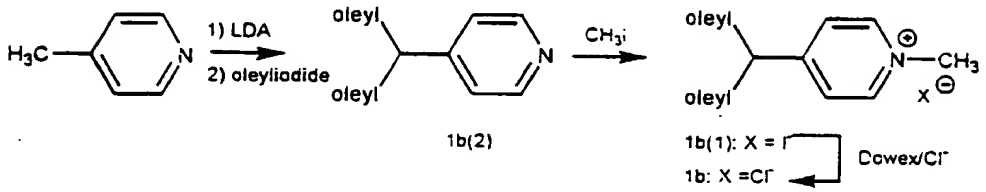


1a(2) 1a

1.2. Synthesis of 1-methyl-4-(19-cis,cis-heptatritiaconta-9,28-dienyl)pyridinium chloride (SAINT-2).

Scheme 2 describes the sequence of the reactions.

Scheme 2:



The synthesis has been carried out under nitrogen. 2.226 g (0.022 mol) of di-isopropyl amine was dissolved in 15 ml of dry diethyl ether. Then 13.8 ml (1.6 M) n-butyl lithium in n-hexane was added dropwise at 0°C. Subsequently, the mixture was stirred for 10 minutes. This mixture was added dropwise to 0.931 g (0.01 mol) 4-picoline in 10 ml of diethyl ether at -20°C. After this it was stirred for another 30 minutes. The colour of the reaction mixture became deeply orange. Then 7.567 g (0.020 mol) oleyl iodide (85% cis) in 5 ml of diethyl ether was added one portion. The temperature increased to 0°C while stirring. Subsequently, the mixture was stirred during one night at room temperature. The next day 100 ml of diethyl ether was added to the reaction mixture and subsequently 40 ml of H_2O. The organic layer was separated and washed with 3 portions of 30 ml H_2O. The ether layer was dried on Na_2SO_4, filtered and condensed. The residu (5.9 g) is a viscous brown oil which was purified over a column of 100 g neutral Al_2O_3 (act. 2-3). As eluent a mixture of n-hexane-diethyl ether (8:2) was used. 4.32 g (0.0073 mol) 4-(19-cis,cis-heptatritiaconta-9,28-dienyl)pyridine was obtained (intermediate 1b2, yield 73%).

NMR data: ^1H NMR($CDCl_3$): δ 0.89 (t, 6H); 1.27 (chain, 52H); 2.0 (m, 8H); 2.43 (tr.1H); 5.34 (m, 4H); 7.06 (d, $J_{H,H}$=6Hz, 2H); 8.49 (d, $J_{H,H}$=6Hz, 2H). ^{13}C NMR: δ 14.0 (CH_3); 22.6; 27.1; 27.3; 29.1; 29.2; 29.4; 29.5; 29.6; 29.7; 31.8; 36.1 (CH_2-chain); 45.5 (CH); 123.1 (CH) 129.7 (CH); 129.8 (CH); 149.5 (CH); 155.3 (C).

1.527 g (0.0025 mol) of intermediate 1 was dissolved in 10 ml of acetone. Subsequently, 2 ml of methyl iodide was added and the mixture was boiled for 3 hours. After evaporation of the solvent a light yellow brown viscous oil was obtained with a yield of 0.8 g (intermediate 1b1, yield 97%).

NMR data: ^1H NMR($CDCl_3$): δ 0.85 (t, 6H); 1.23 (chain, 44H); 1.55 (m, 4H); 1.73 (m, 4H); 2.00 (m, 8H); 2.77 (m, 1H); 4.7 (2, 3H); 5.31 (m, 4H); 7.74 (d, $J_{H,H}$=6.7HZ, 2H); 9.31 (d, $J_{H,H}$=6.7Hz, 2H). ^{13}C NMR: δ 13.9 (CH_3); 22.4; 26.9; 27.2; 28.9; 29.1; 29.3; 29.4; 29.5; 31.6; 35.4 (CH_2-chain); 46.4 (CH); 48.3 (N-CH_3); 126.8 (CH); 129.5 (CH); 129.7 (CH); 144.9 (CH); 167.1 (C).

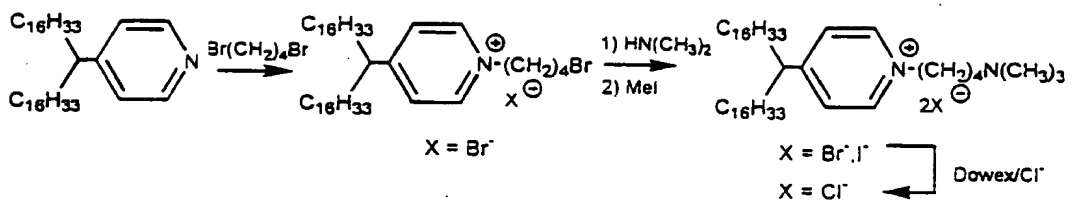
0.4 g (0.00054 mol) of intermediate 2 was dissolved in 3 ml of methanol and this solution was eluted with methanol over a Dowex column (1*8, 200-400 mesh Cl^- form). The compound 1b was obtained as a viscous oil in a yield of 0.319 g (0.00049 mol 92%).

NMR data: ^1H NMR($CDCl_3$): δ 0.87 (t, 6H); 1.26 (CH_2-chain, 44H); 1.57 (m, 4H); 1.75 (m, 4H); 2.00 (m, 8H); 2.77 (m, 1H); 4.77 (s, 3H); 5.32 (m, 4H); 7.15 (d, $J_{H,H}$=6.2Hz, 2H); 9.50 (d, $J_{H,H}$=6.2Hz, 2H).

1.3. Synthesis of 1-(1-butyl-N,N,N-trimethyl ammonium)-4-(17-tritiacontanyl)pyridinium chloride.

This compound was synthesized according to scheme 3 below.

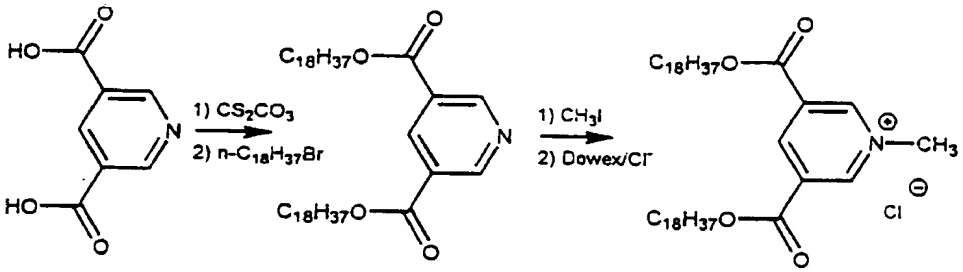
Scheme 3:



2. 3,5-Disubstituted-N-alkylpyridinium salts.

The general synthesis according to scheme 4 below was described in the literature by Sudhölter (vide supra) and Wang et al., J. Org. Chem. 42, 1286 (1977).

Scheme 4:



2.1. 1-methyl-3,5-dicarbo-N-octadecyloxy) pyridinium chloride.

This compound was synthesized according to scheme 4. NMR data: ^{1}H NMR($CDCl_3$): δ 0.85 (t, 6H); 1.30 (chain, 64H); 4.40 (t, 4H); 5.03 (s, 3H); 9.20 (t, 1H); 10.00 (d, 2H).

3. 4-Substituted-N-alkyl pyridinium salts.

The synthesis was described by F.J.A. Hundscheid and J.B.F.N. Engberts, J. Org. Chem. 49, 3088 (1984).

3.1. 1-Methyl-4((-n-hexadecyloxy)carbonyl) pyridinium iodide.

The synthesis of this compound and its characterisation are described by Hundscheid and Engberts (vide supra). NMR data: ^{1}H NMR($CDCl_3$): δ 0.9 (t, 3H); 1.25 (m, 28H); 4.35 (t, 2H); 4.70 (s, 3H); 8.35 (d, 2H); 9.35 (d, 2H).

4. 4-Substituted-N-aralkyl pyridinium salts.

4.1. 1-(3-phenyl-1-propyl)-4-n-dodecylpyridinium iodide.

The compound was synthesized by boiling a mixture of 2.26 g (9.2 mmol) 1-iodo-3-phenyl propane and 2.57 g (10.0 mmol) 4-n-dodecylpyridine in 35 ml of dry acetone for 16 hours. The solvent was evaporated and the yellow solid substance was recrystallized from THF/ether. The yield is 3.06 g (6.2 mmol), melting point 79.0-80.0°C.

NMR data: ^{1}H NMR($CDCl_3$): δ 0.83 (t, 3H); 1.21 (chain, 20H); 1.61 (m, 2H); 2.36 (m, 2H); 2.78 (m, 2H); 4.89 (t, 2H); 7.05-7.20 (m, 5H); 7.73 (d, 2H); 9.30 (d, 2H).

EXAMPLE 2

Formation of unilamellar vesicles

A suitable amount of lipid was dried under N_2(g). In case of combinations of substances these are first mixed and then dried. The lipid film layer is subsequently dried further under vacuum. The lipids are then suspended, vortexed and subsequently sonicated in a suitable volume of water until the solution is clear.

EXAMPLE 3

Transfection of eucaryotic cells by compounds according to the invention

DNA and unilamellar vesicles, as prepared in Example 2, are both brought into Hepes buffered saline (HBS, pH 7.4; both 0.5 ml) and subsequently mixed. The DNA/amphiphile complex is directly formed. In a typical transfection experiment 1 µg of DNA and 7.5-10 µg of the amphiphile SAINT-2 (1-methyl-4-(19-cis,cis-heptatritia contadienyl-9-28) pyridinium chloride) or 1 µg of DNA and 10-15 µg of total amphiphile (SAINT-2/DOPE 1:1) is used.

Cells in six-well plates, which are cofluent by 70-80%, are washed twice with 1 ml of HBS and subsequently 1 ml of the DNA/amphiphile complex was added per well. The cells were incubated during 4 hours at 37°C after which 1 ml Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% Foetal Calf Serum (FCS) was added. After an incubation of 16 hours at 37°C the medium was exchanged by 2 ml fresh DMEM with 10% FCS. After a subsequent incubation of 28 hours at 37°C the cells were gathered. The cells were washed twice with a phosphate buffered saline (PBS) and scraped in 300 µl 1x lysis buffer (Promega). The scraped cells were incubated for 10 minutes at 56°C and subsequently centrifuged at maximum speed for two minutes at room temperature. On the supernatant an enzyme determination (CAT-assay) and a protein determination (Lowry) were carried out.

100 µl of the cell extract was incubated together with 3µl ^{14}C-chloramphenicol (25 mCi/l), 5µl N-butyryl-CoA (2 mg/ml) and 17 µl 0.25 M Tris.HCL (pH 8.0) during 90 minutes at 37°C. The reaction was stopped by adding 0.3 ml of mixed xylenes (Aldrich). The samples were vortexed for 30 seconds and subsequently centrifuged at maximum speed for 3 minutes at room temperature. The organic phase was again extracted with 0.1 ml 0.25 M Tris.HCL, vortexed for 30 seconds and centrifuged for 3 minutes. 4 ml of counting fluid was added to 0.2 ml of the organic phase and the radio activity was measured.

It was found that transfection of COS-7 cells with the new amphiphile (SAINT-2 and SAINT/DOPE) is eight times more efficient that that with DOTMA/DOPE vesicles (see Fig. 1).

It appeared that with the new amphiphile also other cell types, including for instance BHK cells, can be transfected (Fig. 2). When a stable transfection is carried out with the new amphiphile it appeared to be possible to transfect 42-45% of the COS-7 cells. With DOTMA-DOPE vesicles on average 25-29% of the cells are transfected.

EXAMPLE 4

Transport of proteins in an eucaryotic cell

The synthetic amphiphile SAINT-2 is, in combination with DOPE, a suitable agent for the delivery of proteins into cells. The efficiency of protein internalisation with SAINT-2/DOPE as a carrier can be monitored with the aid of the gelonine protein. Internalized gelonine specifically inhibits the protein synthesis of cells and this inhibitation is a direct measure for the amount of gelonine which has been brought into the cell. Unilamellar versicles of the synthetic agent amphiphile SAINT-2 and DOPE are obtained by bath sonication. Gelonine is added to a certain concentration (0-20µM) SAINT-2/DOPE in HBS from a stock solution (2 mg/ml).

CV-1 cells, grown in twelve-well plates, are washed three times with HBS. Subsequently, the cells are incubated for 1 hour at 37°C with the amphiphile/gelonine complex in HBS obtained in this way. After this the cells are again washed three times with HBS.

The inhibition of protein synthesis by gelonine is being followed by determining the building-in of radioactively labelled methionine into the treated cells. This is carried out by incubating the cells for 30 minutes with 1 µCi ^{35}S-methionine. Subsequently, the cells are washed three times with PBS and finally scraped in 10% TCA. The cell lysate obtained in this way is washed three times with 10% TCA and the amount of radioactive methionine present in the cell lysate is determined with the aid of a scintillation counter.

Incubation of CV-1 cells with the amphiphile/gelonine complex gives a strong inhibition of the protein synthesis with respect to the control experiment in which the cells were incubated with the synthetic amphiphile only. At a concentration of 5 µM SAINT-2/DOPE and 1.6 µM gelonine an inhibition of protein synthesis of 50% was obtained.

EXAMPLE 5

Toxicity studies

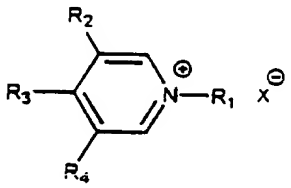
To determine the toxicity of the compound SAINT-2 according to the invention with respect to DOTMA-DOPE the COS-7 cells are incubated with different concentrations of both lipid samples. The residual protein content is taken as a measure for the amount of surviving cells.

A decrease of the protein content from 2 to 1 mg/ml was observed for DOTMA-DOPE starting from 71 μM lipid. For SAINT-2 a decrease from 2 to 1.75 mg/ml was found starting from 90 μM.

This shows that SAINT-2 is clearly less toxic than DOTMA-DOPE.

Claims

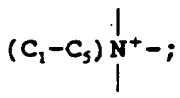
1. Compounds with the general formula I



(I)

in which:

R_1 is a (C_1-C_5)alkyl, ar(alkyl) or an alkyl group with a cationic functional group, like

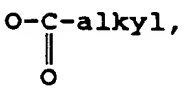


or

R_1 is (C_1-C_5) R_5 in which R_5 is a structure with the general formula I;

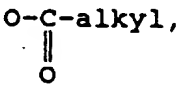
X is a halide counter ion; chosen from Cl^-, I^-, Br^-; and in which

or R_3 is hydrogen and R_2 and R_4 are identical or different and are chosen from the group, comprising branched or linear (C_{10}-C_{20})alkyl, a mono- or polyunsaturated (C_{10}-C_{20})alkenyl, O=C-O-alkyl,



or ar(alkyl),

or R_2 and R_4 are hydrogen and R_3 is -$CH(R_5)_2$ with R_5, comprising (C_{10}-C_{20})alkyl, mono- or polyunsaturated (C_{10}-C_{20})alkenyl, O=C-O-alkyl,



or aralkyl,

wherein disclaimed are the compounds with the general formula I in which R_1 is CH_3, R_2 and R_4 are hydrogen, R_3 is $(C_{16}H_{33})_2CH$ and X is all mentioned counter ions (Cl^-, I^-, Br^-) and disclaimed are the compounds in which R_1 is

CH_3, R_2 and R_4 are $C_{16}H_{33}$-O-C(O), R_3 is hydrogen and X is all mentioned counter ions (Cl^-, I^-, Br^-).

2. Compounds according to claim 1, **characterized** in, that R_1 is CH_3, R_2 and R_4 are hydrogen, R_3 is $(C_{18}H_{37})_2CH$ and X is Cl^-, Br^-, I^-.

3. Compounds according to claim 1, **characterized** in that R_1 is CH_3, R_2 and R_4 are hydrogen, R_3 is $(C_{18}H_{35})_2CH$ and X is Cl^-, Br^-, I^-.

4. Compounds according to claim 1, **characterized** in that R_1 is $(CH_2)_4$-$N^+(CH_3)_3$, R_2 and R_4 are hydrogen, R_3 is $(C_{18}H_{35})_2CH$ and X is Cl^-, Br^-, I^-.

5. Compounds according to claim 1, **characterized** in that R_1 is CH_3, R_2 and R_4 are $C_{18}H_{37}$-O-C(O), R_3 is hydrogen and X is Cl^-, Br^-, I^-.

6. Compounds according to claim 1, **characterized** in that R_1 is CH_3, R_2 and R_4 are R_5-O-C(O), in which R_5 is a saturated or unsaturated C_{10}-C_{20} aliphatic chain or aralkyl, R_3 is hydrogen and X is Cl^-, Br^-, I^-.

7. Compounds according to claim 1, **characterized** in that R_1 is CH_3, R_2 and R_4 are not identical and each represents a group with the formula R_5-O-C(O), in which R_5 is a C_{10}-C_{20} alkyl, aralkyl, alkenyl or polyunsaturated alkyl is, R_3 is hydrogen and X is Cl^-, Br^-, I^-.

8. Compounds according to claim 1, **characterized** in that R_1 is CH_3, R_3 is a group with the formula R_5-O-C(O), in which R_5 is a C_{10}-C_{20} alkyl, aralkyl, alkenyl or polyunsaturated alkyl, R_2 and R_4 are hydrogen and X is Cl^-, Br^- or I^-.

9. Compounds according to claim 8, **characterized** in that R_5 is $C_{16}H_{33}$.

10. Compounds according to claim 10, **characterized** in that R_1 is $(CH_2)_n$-C_6H_5, in which n = 3-6, R_2 and R_4 are hydrogen, R_3 is alkyl or alkenyl and X is Cl^-, Br^- or I^-.

11. Compound according to claim 10, **characterized** in that R_1 is $(CH_2)_3$-C_6H_5, R_2 and R_4 are hydrogen, R_3 is n-$C_{12}H_{25}$ and X is Cl^-, Br^- or I^-.

12. Compounds according to claim 1, **characterized** in that R_1 is $(CH_2)_4$, R_2 and R_4 are hydrogen, R_3 is $(C_{18}H_{35})_2CH$, X is Cl^-, Br^-, I^- and R_5 is the group with the general formula I, bound through R_1, in which R_2 and R_4 are hydrogen, R_3 is $(C_{18}H_{35})_2CH$ and X is Cl^-, Br^-, I^-.

13. Compounds according to one of the preceding claims for use as tool to introduce macromolecules into cells.

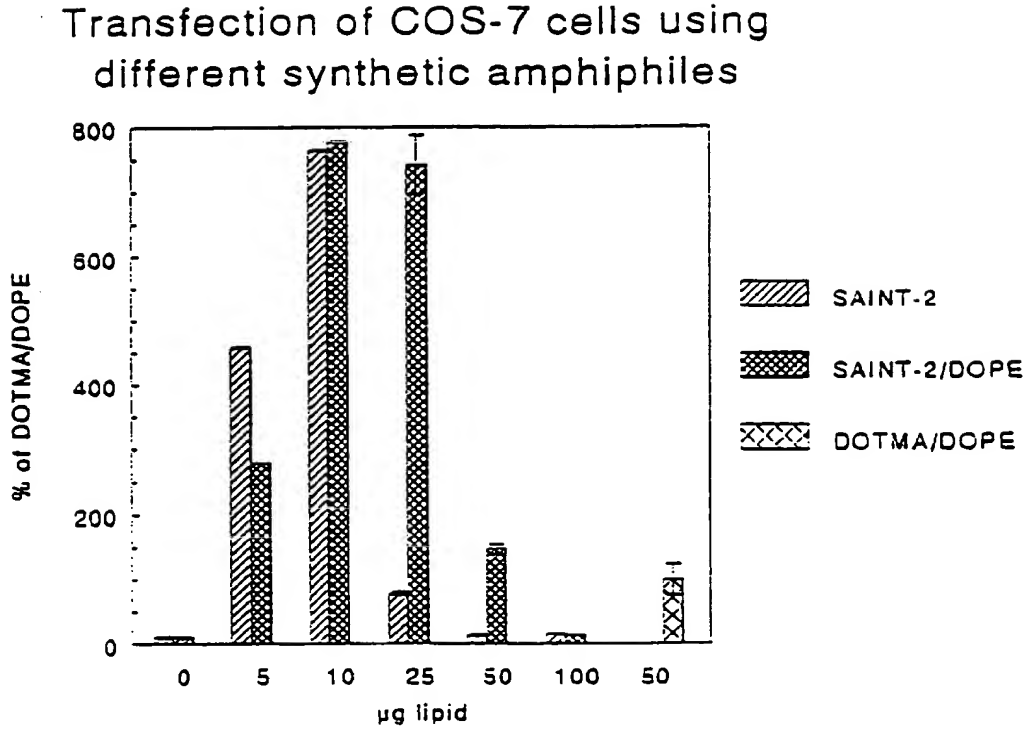
14. Composition to introduce macromolecules into cells, comprising vesicles or another type of aggregate formed by at least one compound according to one of the preceding claims in a solvent.

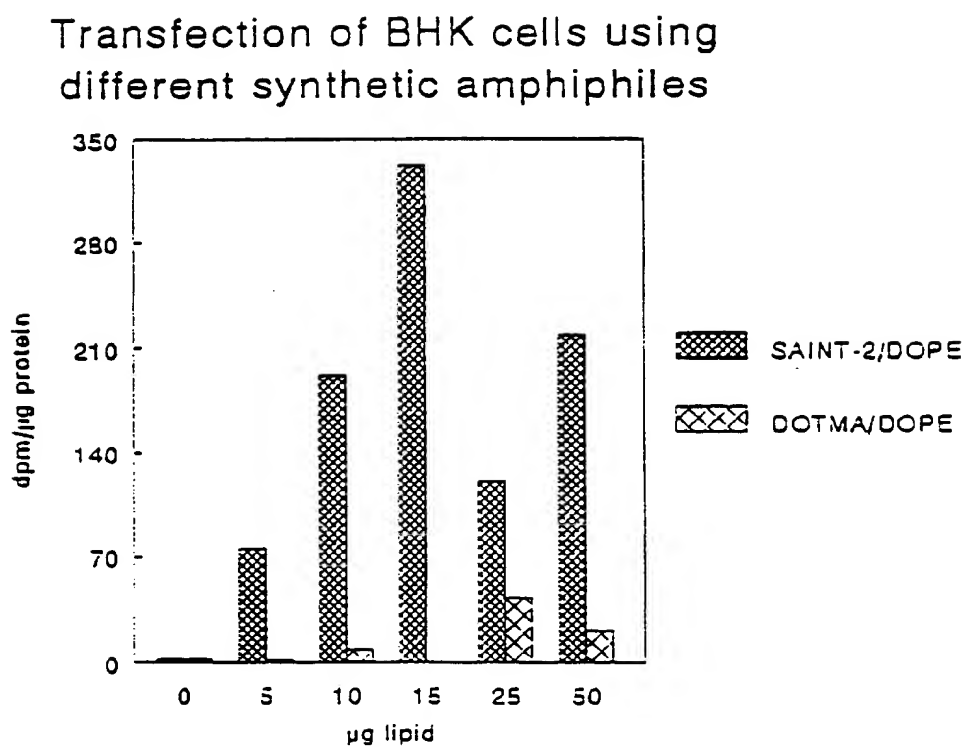
15. Composition according to claim 14, **characterized** in that at least one macromolecule is incorporated in and/or attached to the vesicles.

16. Composition according to claim 14 or 15, **characterized** in that furthermore at least one targeting molecule is attached to the vesicles.

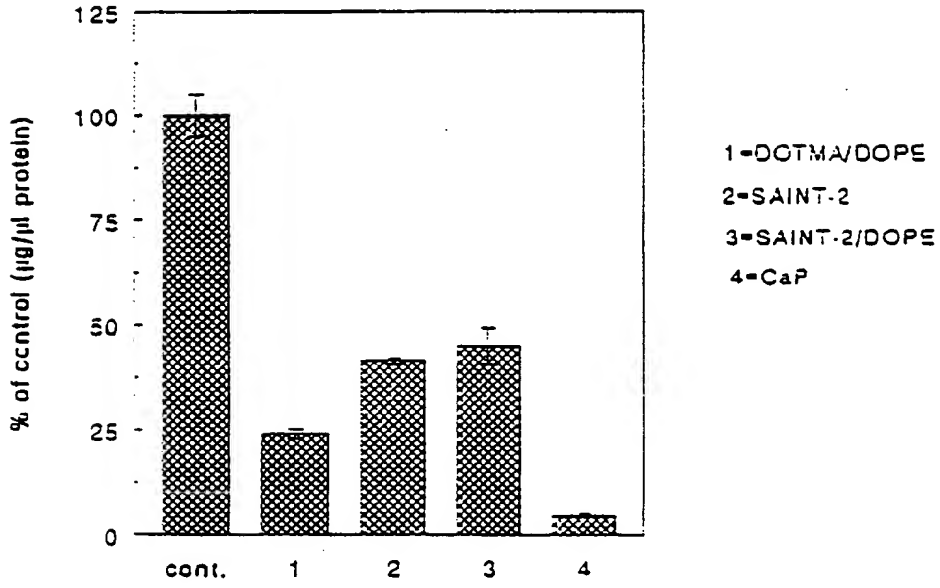
17. Composition according to claim 15, **characterized** in that the targeting molecule is an antibody.

18. Composition according to claim 16, **characterized** in that the antibody is radioactively labelled or labelled with streptavidine.


Transfection of COS-7 cells using different synthetic amphiphiles
% of DOTMA/DOPE
800
600
400
200
0
0
5
10
25
50
100
50
µg lipid
SAINT-2
SAINT-2/DOPE
DOTMA/DOPE


Transfection of BHK cells using different synthetic amphiphiles
350
280
210
140
70
0
dpm/µg protein
0
5
10
15
25
50
µg lipid
SAINT-2/DOPE
DOTMA/DOPE

Vesicle-mediated versus CaP-mediated transfection



European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 96 20 2095

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	EP-A-0 516 194 (PUETTER MEDICE CHEM PHARM) 2 December 1992 * the whole document * ---	1-18	C07D213/20 A61K31/44
X	CHEMICAL ABSTRACTS, vol. 112, no. 1, 1 January 1990 Columbus, Ohio, US; abstract no. 7383s, N.S. BODOR : "Preparation of dihydropyridine-containing prodrugs for brain-specific drug delivery." page 724; XP002017482 zie CAS RN 89722-34-9 * abstract * & US-A-4 824 850 (UNIVERSITY OF FLORIDA) ---	1,6, 14-18	
X	CHEMICAL ABSTRACTS, vol. 96, no. 22, 31 May 1982 Columbus, Ohio, US; abstract no. 191111b, E.J.R. SUDHOLTER ET AL.: "Thermotropic liquid-crystalline behavior of some single- and double-chained pyridinium amphiphiles." page 703; XP002017483 zie CAS RN 73570-94-2 en 80243-89-6 * abstract * & JOURNAL OF PHYSICAL CHEMISTRY, vol. 86, no. 10, 1982, WASHINGTON, D.C., pages 1908-1913, -----	14-18	

TECHNICAL FIELDS SEARCHED (Int.Cl.6)
C07D
A61K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	4 November 1996	Bosma, P

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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